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A convenient spectroscopic method for the estimation of hemoglobin concentrations in cell-free solutions

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Summary

Using a millimolar absorptivity of 7.12 ± 0.09 at 523 nm, it is possible to estimate the total concentration of hemoglobin in solutions containing oxyhemoglobin, deoxyhemoglobin and methemoglobin in any combination. This estimate is independent of the pH in the range 6.0–10.0 and will provide concentrations comparable to that obtained by the conventional and more precise use of cyanomethemoglobin. This methodology should be of value for the determination of extracellular hemoglobin in vivo, as a means for determining the vascular half-life of stroma-free hemoglobin based blood substitutes.

Key words: Methemoglobin spectrum; Isobestic point; pH dependency; Concentration estimation; Hemoglobin

Introduction

Hemoglobin concentrations are routinely estimated by conversion to the cyanomethemoglobin derivative [1] which is a stable derivative with a broad maximum centered at 540 nm and a millimolar absorptivity of 11.00 [2]. By this procedure, it is possible to determine the total hemoglobin content of solutions which contain mixtures of oxyhemoglobin, deoxyhemoglobin, methemoglobin and

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carboxyhemoglobin with an error of less than 2%. Another minor component occasionally present is sulfohemoglobin which is not converted to the cyanomethemoglobin, but it causes little error since it is normally present in low concentration and it has a millimolar absorptivity of approximately 7 at 540 nm. It is also possible to approximate the hemoglobin concentration by using a millimolar absorptivity of 15.3 at 576 nm [3] on the basis that the major component of an aerated solution is composed mainly of oxyhemoglobin.

For the study of the metabolic fate of in vivo stroma-free hemoglobin, however, it can be anticipated that the solution would contain a mixture of oxy-, deoxy- and methemoglobin. Estimation of the total concentration by cyanomethemoglobin is awkward, and the estimation at 576 nm would be erroneous. This error would be further enhanced by the pH dependence of the methemoglobin spectra.

During a study of the formation of methemoglobin as a function of pH, it was observed that isobestic points or a narrow isobestic region occurred at 489, 523 and 616 nm. An isobestic point near 523 nm had also been observed by Kaplan [4] who used it to calculate the amount of methemoglobin in solutions of oxyhemoglobin. However, both oxy- and deoxyhemoglobin have pH independent absorbances at 523 nm which were identical to that of equal concentrations of methemoglobin. Since neither carboxy- nor sulfohemoglobin is expected to be formed in these studies, a convenient and rapid method for the estimation of total hemoglobin concentration was suggested.

Experimental

Stroma-free hemoglobin (pH 7.2, 10 μS) was prepared from recently outdated human blood by the method of Rabiner et al. [5]. Oxygenated hemoglobin was used directly since no spectral change was observed after equilibration with water-saturated oxygen in a tonometer. The solutions for spectral analysis were prepared from a common stock of stroma-free hemoglobin (8 g/dl, with $A_{577\,nm} = 76$ determined on a 1 : 100 dilution at 1 cm lightpath) by passing 10 ml through a mixed-bed ion-exchange column of Bio-Rex RG 501-X8 (1.5×30 cm) and capturing it in a 50 ml volumetric flask. The ion free eluate (< 5 μS) was diluted 1 : 10 with 0.11 M Tris-HCl, pH 7.4, and the spectra taken with a Cary-14 spectrophotometer equipped with an on-line data acquistion system (OLIS, Jefferson, GA). Spectra were obtained between 450 and 650 nm at intervals of 1 nm. Each spectrum was taken at least twice and averaged.

Deoxyhemoglobin was prepared from this solution by placing 4 ml in a tonometer (Instrumentation Lab. Inc., Lexington, MA) and equilibrating with water-saturated nitrogen at room temperature for 15 min. Samples were taken directly from the tonometer with a nitrogen-purged syringe and transferred to a nitrogen-purged teflon-stoppered cuvette and the spectra obtained. Sodium dithionite crystals were then added and the spectra again recorded. Occasionly there were slight alterations observed, and only those spectra which were essentially unchanged by the addition of dithionite were considered. Longer periods for the conversion to

deoxyhemoglobin in the tonometer caused alterations in the hemoglobin concentration.

Carboxyhemoglobin was prepared from the dilute deionized oxyhemoglobin by passing water-saturated carbon monoxide over a stirred solution at room temperature for periods up to 1 h. The solutions were transferred with a carbon monoxide-purged syringe into a carbon monoxide-purged teflon-stoppered cuvette and the spectrum recorded. Complete conversion to carboxyhemoglobin required longer times than that for conversion to deoxyhemoglobin so the spectra obtained were altered due to the differences in concentration. Adjustment of these spectra on the basis of the concentrations determined by the cyanomethemoglobin derivative gave a millimolar absorptivity of 10.1 at 523 nm.

Methemoglobin was prepared from an equivalent solution of the original hemoglobin by using 1.2 equivalents of $K_3Fe(CN)_6$ or 4 equivalents of $NaNO_2$ per heme. An excessive amount of nitrite caused the formation of $HbNO_2$ and an anomalous spectrum which, however, was converted to the normal methemoglobin spectrum by the removal of the excess reagent on the mixed-bed column. After reacting for 1 h in the dark, 10 ml of each solution was passed through the mixed-bed resin column and captured in 50 ml volumetric flasks. These solutions had conductivities of less than 5 μS, and they were diluted 1 : 10 with 0.11 M Tris-HCl at the desired pH. After passage through the column, the ferricyanide samples were partially converted to oxyhemoglobin ($\simeq 3\%$) and some protein was lost. The nitrite-treated samples were resistant to this reduction and were present as methemoglobin with no loss of protein. These spectra were used for the analysis. The ferricyanide-oxidized samples were used for the analysis of concentrations in mixed solutions.

Various mixtures of oxy- and methemoglobin were prepared from the column eluates ($\simeq$ 1 mM) and diluted 1 : 10 with 0.11 M Tris-HCl at the desired pH. These samples were at the same total concentration as those previously prepared, and the spectra, as well as the total concentration of hemoglobin in the solutions [1] and the methemoglobin concentration [6], were obtained. Methemoglobin was also estimated by an analysis of the spectra by a 200 point inversion matrix [7].

Results and Discussion

Spectra of methemoglobin as a function of pH from 6.1 to 10.22 at room temperature in 0.1 M Tris are shown in Fig. 1. The isobestic point or region at 523 nm has an absorption of 7.12 ± 0.09 for a millimolar concentration of methemoglobin. The values ranged from 7.09 to 7.42 and the higher values all occurred above pH 9.4. This increase is also present in the calculation of the ionization constant for the ionization of ferriheme (Fig. 2) and may be due to a general unfolding at elevated pH. The pK' calculated for a variety of wavelengths is 7.99 ± 0.08 using the curve-fitting algorithm, MLAB [8]. A pK' value of 8.14 had previously been reported for $\mu = 0.05$ at 20 °C [9]. Brunori et al. [10] found p$K' = 8.05$ at 20 °C and $\mu = 0.2$. The isobestic point at 523 nm may also be seen for the spectra of oxy- and deoxyhemoglobin (Fig. 3). The curves were obtained on

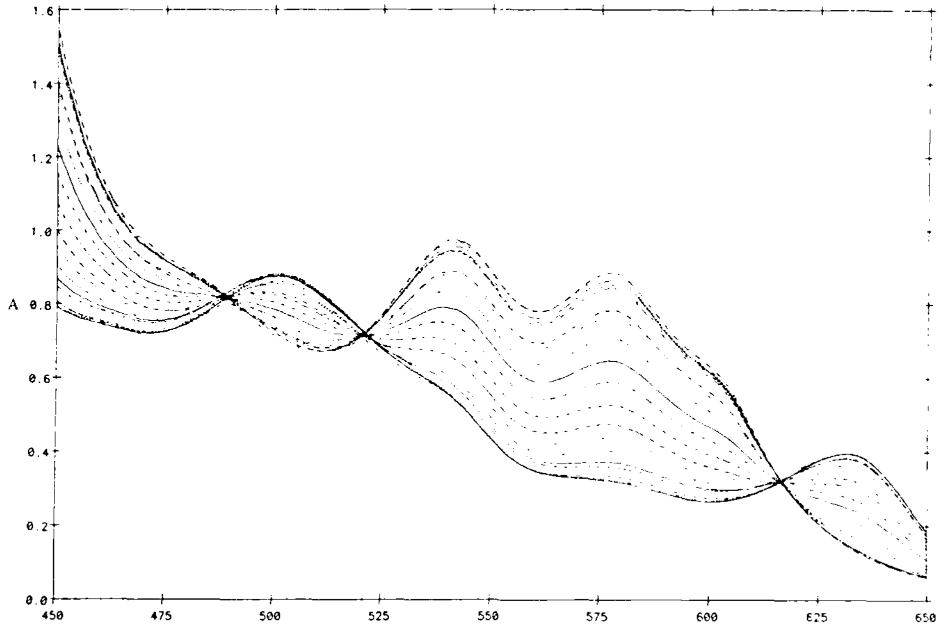

Fig. 1. Absorbance of methemoglobin as a function of pH. The lowest curve at 579 nm is that obtained at pH 6.1 in 0.1 M Tris-HCl. Subsequent spectra to pH 10.22 are those obtained at approximately 0.2 pH intervals. All the spectra were obtained at room temperature. The concentration of the samples was 0.1 mM.

TABLE 1

MILLIMOLAR ABSORPTIVITIES OF HEMOGLOBIN DERIVATIVES

Lambda	HbO_2	SD	Hb	SD	MetHb	SD
450	15.88	0.10	14.57	0.20	9.87	0.05
460	10.88	0.05	5.37	0.17	8.58	0.10
470	8.15	0.03	3.58	0.13	7.96	0.09
480	6.55	0.03	3.42	0.11	7.95	0.10
490	5.63	0.03	3.73	0.09	8.37	0.12
500	5.10	0.03	4.42	0.08	8.59	0.13
510	4.85	0.04	5.47	0.07	8.12	0.11
520	6.03	0.04	6.58	0.07	7.38	0.06
530	10.48	0.04	8.16	0.11	6.97	0.11
540	14.22	0.02	10.67	0.16	6.72	0.20
550	11.57	0.05	13.03	0.11	5.55	0.17
560	8.58	0.04	13.06	0.11	4.57	0.16
570	11.89	0.06	11.39	0.09	4.63	0.21
580	13.70	0.07	9.13	0.18	4.67	0.22
590	3.64	0.10	6.76	0.05	3.95	0.06
600	0.86	0.06	3.65	0.06	3.45	0.10
610	0.38	0.05	1.97	0.05	3.21	0.10
620	0.22	0.05	1.41	0.04	3.30	0.09
630	0.15	0.05	1.18	0.05	3.48	0.10
640	0.11	0.05	1.04	0.04	2.90	0.09
650	0.08	0.05	0.96	0.04	1.57	0.06

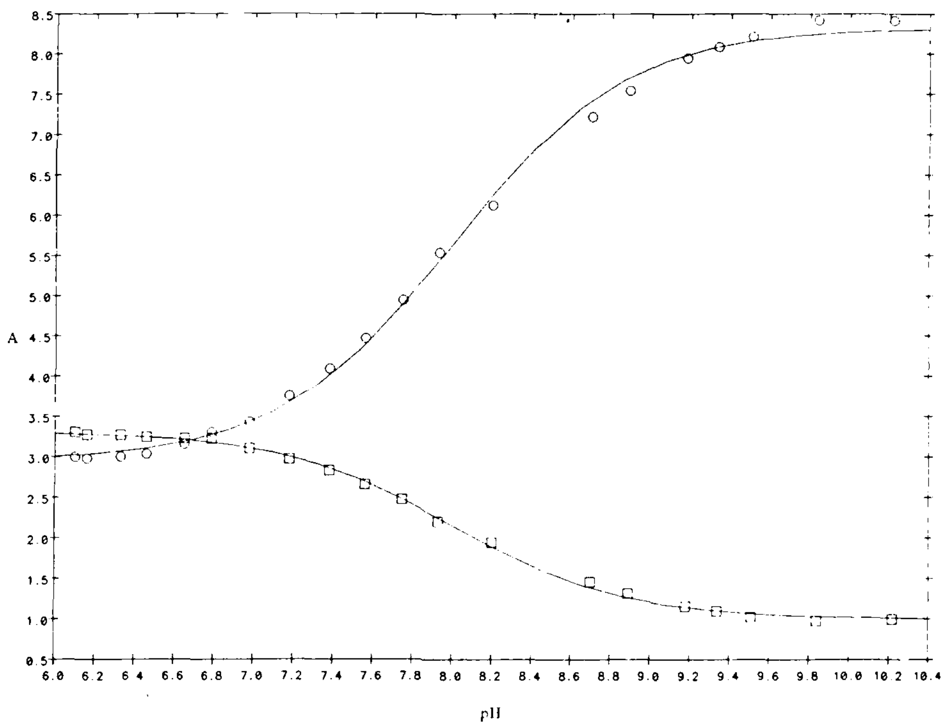

Fig. 2. The pH dependence of the spectra of methemoglobin. Points are the experimental values expressed as the millimolar absorptivity differences; the lines are calculated for a pK' of 7.99 for the ionization of ferriheme. The upper curve (circles) is obtained at 579 nm; the lower curve (squares) is that found at 639 nm.

solutions prepared by the volumetric procedures described. They may also be obtained by conversion of any concentration of hemoglobin using the millimolar absorptivities calculated from the absorption at 523 nm, or by the absorption of the cyanomethemoglobin at 540 nm using a millimolar absorptivity of 11.0 [3]. From a minimum of 17 separate spectra, the average absorptivities and the standard deviation were determined and are given in Table 1.

The millimolar absorptivity for HbO_2 found is somewhat below that of Van Assendelft [13], ϵ_{577} of 15.37. It is, however, somewhat higher than the values reported by Zijlstra et al. [14] for HbO_2 solutions of the concentration 0.05 mM which is comparable to those used in the present study (0.03–0.1 mM). They reported a millimolar absorptivity at 577 nm of 15.03 ± 0.27 compared to 15.22 ± 0.08 found here.

It is difficult to compare the spectra of methemoglobin found here with that given by Van Assendelft [13] inasmuch as he reported the spectra for the pH range 7.0–7.4 to be identical. The spectra shown in Fig. 3 indicate that the spectra are pH-dependent in this range. The values reported (Table 1) tend to fall between those of Van Assendelft [13] and Benesch et al. [12]. This variation is not due to the

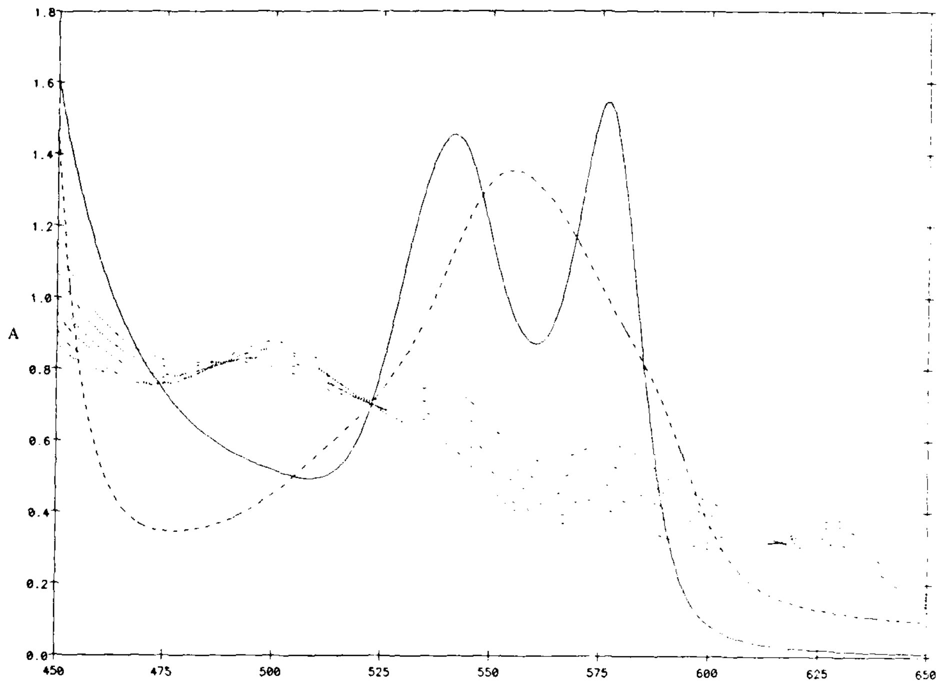

Fig. 3. Isobestic regions for the spectra of oxyhemoglobin (solid line), deoxyhemoglobin (dashed line) and methemoglobin. The methemoglobin spectra (dotted lines) are for pH 6.98 (lower curve at 575 nm) and pH 7.21, 7.41, 7.55 and 7.80, successively. The concentrations of the samples were prepared as discussed in the text to be 0.1 mM so that the millimolar absorptivities may be easily calculated.

nature of the buffers used inasmuch as spectra in phosphate buffers (0.1 M) for the pH values ranging from 6.0 to 8.0 are identical to those in 0.1 M Tris-HCl. Nor are the variations due to any differences in the methemoglobin structure caused by either ferricyanide or nitrite since these spectra are very nearly identical after passage through the mixed-bed resin when the slight reversion to oxyhemoglobin is considered. Nonetheless, because of the difficulty encountered in preparing a fully oxidized preparation that is also stripped of the oxidizing reagent, the spectra show a greater variation then either the oxy- or deoxyhemoglobin spectra. For deoxyhemoglobin, a millimolar absorptivity $\epsilon_{555\,nm}$ of 13.35 ± 0.12 is found. This compares to $\epsilon = 13.29$ reported by Zwart et al. [15] but is higher than $\epsilon = 13.04$ reported by Van Assendelft [13] at the same wavelength.

A comparison of the estimation of hemoglobin concentration by the cyanomethemoglobin methodology and the use of the isobestic point at 523 nm is given in Table 2. Concentrations are calculated by:

$$g/dl = (A_{523}\ nm)(dil)(1.611)/7.12 \qquad (1)$$

where the factor 1.611 converts millimolar to gram per decilitre. Various concentra-

TABLE 2

COMPARISON OF HEMOGLOBIN CONCENTRATION BY DRABKIN'S AND BY THE ABSORPTION AT 523 nm

Hb (g/dl) (Drabkin's)	Hb (g/dl) (A_{523nm})	MetHb (%)
0.93	1.01	4.50
0.93	1.01	11.10
1.36	1.37	4.22
1.46	1.54	4.40
1.61	1.60	1.40
1.76	1.76	8.49
2.00	2.00	5.40
7.77	7.85	1.70
7.91	7.85	100.00
8.02	7.93	10.82
9.33	9.35	0.64
9.62	9.63	100.00
9.88	9.71	1.66
13.60	13.33	9.10

tions of hemoglobin containing the fractions of methemoglobin shown were analyzed with very good agreement. Some of the lower concentration samples were exposed to water-saturated nitrogen for varying periods of time to provide some deoxyhemoglobin. The amount of deoxyhemoglobin present was not monitored.

TABLE 3

VARIATION OF THE HEMOGLOBIN CONCENTRATION BY ESTIMATION AT 523 nm[a]

Absorption	Hb (g/dl)
0.6518	7.37
0.6531	7.39
0.6765	7.65
0.6775	7.66
0.6718	7.60
0.6933	7.84
0.6571	7.43
0.6896	7.80
0.7137	8.07
0.6848	7.75
0.6971	7.89
0.6961	7.87
0.6790	7.68
0.6946	7.86
0.6868	7.77
Mean 0.6841	7.74
SD 0.0166	0.19

[a] The original hemoglobin solution contained 0.66% methemoglobin. Some samples were converted to methemoglobin by ferricyanide or nitrite, deionized, diluted and assayed. Others were converted partially to deoxyhemoglobin and assayed.

TABLE 4

ESTIMATION OF HEMOGLOBIN CONCENTRATION AT VARIOUS pH VALUES USING A_{523nm}

pH	A_{523nm}	Hb (g/dl)
5.96	0.8589	9.72
6.17	0.8788	9.94
6.44	0.8525	9.64
7.01	0.8631	9.76
7.21	0.8601	9.73
7.41	0.8621	9.75
7.55	0.8605	9.74
7.62	0.8529	9.65
7.80	0.8664	9.80
8.43	0.8672	9.81
8.83	0.8633	9.77
9.22	0.8668	9.81
9.59	0.8658	9.79
9.77	0.8655	9.79
9.95	0.8656	9.79
Mean	0.8630	9.76
SD	0.0060	0.07

Using a single sample of stroma-free hemoglobin, the concentrations shown in Table 3 are obtained. The range of concentrations is 7.37 to 8.07 g/dl which seems quite large. These same samples were assayed by the cyanomethemoglobin method which gave a range of 7.46 to 7.99 g/dl with an average of 7.83 ± 0.15 in substantial agreement with the average 7.74 ± 0.19 obtained using the isobestic absorption. This variation may be attributed to the manipulation of the samples during oxidation or deoxygenation and subsequent passage through the mixed-bed ion-exchange column. When a single sample of stroma-free hemoglobin prepared with 10% methemoglobin is analyzed as a function of pH, the values in Table 4 are obtained. The average is 9.76 ± 0.07 which is a better indication of the precision of the method.

It is quite important that the wavelength be precisely defined since the millimolar absorptivity for HbO_2 in this region is rising at 0.31/nm; Hb is rising at 0.12/nm, while that of methemoglobin is relatively flat, varying at −0.04/nm. Notwithstanding this difficulty, it would appear that the millimolar absorptivity at 523 nm is a convenient, precise and relatively simple measurement for estimating total hemoglobin concentrations of solutions consisting of oxy-, deoxy- and methemoglobin.

Simplified description of the method

Samples of hemoglobin solutions are read in a Cary-14 spectrophotometer at 523 nm. This reading is converted to the millimolar concentration by:

$$mM(Hb) = (A_{523nm})(dil)/7.12$$

where 7.12 is the millimolar absorptivity for oxy-, deoxy- and methemoglobin at room temperature in 0.1 M ionic strength and in the pH range 6.0 to 10.

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17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	methemoglobin spectra, isobestic points, pH dependency, estimation of Hb concentration

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Using a millimolar extinction coefficient of 7.12 at 523 nm, it is possible to estimate the total concentration of hemoglobin in solutions containing oxyhemoglobin, deoxyhemoglobin and methemoglobin in any combination. This estimate is independent of the pH in the range 6.0 to 10.0 and will provide concentrations comparable to that obtained by the conventional and more precise use of cyanomethemoglobin. This methodology should be of value for the determination of extracellular hemoglobin *in vivo*.

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